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14. ABSTRACT

The goal of this work was to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism and to optomize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants. Specifically, we found and clined a gene that encodes an enzyme capable of degrading diethylphosphate, (2) cloned and expressed a pathway for complete mineralization of pnitrophenol phosphate, (3) cloned and expressed a phosphotriesterase capable of hydrolyzing parathion, (4) developed a co-culture biofilm capable of degrading parathion (as a proof-of-concept), and (5) combined all of the genes in a single organism for complete mineralization of parathion of paraoxon.

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Final Report

GRANT #: N00014-99-1-0182

PRINCIPAL INVESTIGATOR: Dr. Jay D. Keasling

INSTITUTION: University of California at Berkeley

GRANT TITLE: Strategies for Metabolic Engineering of Environmental Microorganisms - Application to Degradation of Organophosphate Contaminants

AWARD PERIOD: 11/30/1998 - 11/29/2001

OBJECTIVE: The goal of this work is to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism and to optimize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants. The objectives of this work are: (1) to find and clone a gene that encodes an enzyme capable of degrading diethylphosphate, (2) to clone and express a pathway for complete mineralization of *p*-nitrophenol phosphate, (3) to clone and express a phosphotriesterase capable of hydrolyzing parathion, (4) to develop a co-culture biofilm capable of degrading parathion (as a proof-of-concept), and (5) to combine all of the genes in a single organism for complete mineralization of parathion or paraoxon.

APPROACH: Metabolic engineering offers the opportunity to expand the role of bioremediation. Traditional metabolic engineering involves overexpression of a desired protein and leads to a high metabolic burden on the cell. The purpose of this work is to develop strategies to help reduce this burden and make an engineered organism more environmentally effective.

Parathion (O,O-diethyl-O-*p*-nitrophenyl phosphorothioate), an organophosphate pesticide which has been widely used and is highly toxic, was chosen as the model compound for this project. Parathion is also structurally and functionally similar to many chemical warfare agents (including VX and soman).

Andy Walker, a graduate student working on the project, has constructed a strain of *Pseudomonas putida* utilizing paraoxon as a carbon and energy source. The initial step in parathion degradation, parathion hydrolysis, results in the formation of diethyl thiophosphate (DETP) and *p*-nitrophenol (PNP). Sundiep Tehara, another graduate student working on the project, has cloned and expressed a novel phosphodiesterase. The gene for this novel phosphodiesterase will be placed into the Pseudomonad for complete degradation of paraoxon. Eric Gilbert, a post-doctoral research associate, developed a co-culture biofilm capable of degrading parathion and using it as a carbon and energy source. Stacie Cowan, a graduate student, developed a method to track multiple organisms in a biofilm to observe their interactions during degradation of organophosphates.

ACCOMPLISHMENTS: We have (1) characterized an enzyme capable of degrading diethylphosphate and have cloned and expressed the gene encoding the enzyme, (2) expressed in *Pseudomonas putida* the genes encoding a pathway for complete mineralization of *p*-nitrophenol phosphate, (3) cloned and expressed a phosphotriesterase capable of hydrolyzing parathion, (4) developed a co-culture biofilm capable of degrading parathion, and (5) combined all of the genes in a single organism for complete mineralization of parathion.

Pseudomonas putida KT2442 has been engineered to utilize the organophosphate pesticide parathion, a compound similar to other organophosphate pesticides and chemical warfare agents, as a source of carbon and energy. The initial step in the engineered degradation pathway was its hydrolysis by organophosphate hydrolase (OPH) to *p*-nitrophenol (PNP) and diethyl thiophosphate (DETP), compounds that cannot be metabolized by *P. putida* KT2442. Genes encoding OPH (*opd*), with and without the secretory leader sequence, were introduced into the broad-host-range plasmids pMMB206 and pVLT35 and the resulting plasmids were transformed into *P. putida* and *Escherichia coli*. Expression of the leader sequence-free *opd* was highest in *E. coli*; however, little or no OPH activity was observed in *P. putida* when either the modified or native *opd* was expressed from the *taclac* promoter. When the native *opd* gene, containing the secretory leader sequence, was expressed under the control of the *tac* promoter in *P. putida*, high OPH activity was observed. A plasmid carrying an operon encoding the enzymes for PNP transformation to □-ketoadipate, a metabolite utilized by *P. putida* as a source of carbon and energy, was transformed into *P. putida* allowing the organism to utilize 0.5 mM PNP as a carbon and energy source. Transformation of *P. putida* with a plasmid expressing the native *opd* and the plasmid carrying the PNP operon allowed the organism to utilize 0.8 mM parathion as a source of carbon and energy. Degradation studies showed that parathion formed a separate DNAPL phase but was still bioavailable. These results demonstrate the potential of metabolically engineered microorganisms to be used in organophosphate pesticide remediation and chemical warfare disposal.

Organophosphates used as pesticides and chemical warfare agents pose a risk to the environment. Recently, using bis(p-nitrophenyl)phosphate as a substrate, we identified a novel phosphodiesterase (PDE) activity in *Comamonas acidovorans* that could be used in organophosphate biodegradation. In contrast to bacterial alkaline phosphatase, the PDE was also found to have activity against a variety of different p-nitrophenyl substrates. The PDE was purified from a cell lysate and the N-terminal sequence was determined. Southern blot analysis with the N-terminus was completed on a ClaI-digested genomic library. A partial genomic library was constructed with a 7-kb fragment. This fragment was ligated into pBluescript and transformed into *E. coli* DH10B. Colony hybridization was used to screen the library. A positive clone was found and confirmed by sequencing. The sequence showed weak homology to other phosphodiesterases. This clone was placed under the control of the T7 promoter in pET-41b+ and expressed in BL21(DE3).

A technique has been developed to selectively attach bacteria to solid supports using poly-L-lysine. The patterned biofilms were labeled with green fluorescent protein or a nucleic acid stain and imaged using both

confocal microscopy and GFP stereomicroscopy. *E. coli* DH10B, *E. coli* MC1061, and *Pseudomonas* sp. GJ1 were selectively attached to regions coated with poly-L-lysine but not to uncoated regions. In contrast, *E. coli* DH5α, W3110, and 33456 attached indiscriminately to the coated and uncoated regions of the surface. Those organisms that selectively attached to the poly-L-lysine coated regions formed biofilms twice as thick as the organisms that attached indiscriminately to the surface. This technique can be used for selectively patterning surfaces with genetically engineered microorganisms for biosynthesis of secondary metabolites and biodegradation or for developing a bacterial-based microscale medical diagnostic tool.

We have developed a dual labeling technique involving two GFP variants that is compatible with confocal microscopy. Using two lasers, (i) mixed cultures of cells, where one species contained GFPuv and another species contained GFPmut2 or GFPmut3 and (ii) a single species containing both GFPuv and GFPmut2 in the same cell were imaged. This method shows promise for monitoring gene expression and as a nondestructive and in situ technique for confocal microscopy of multispecies biofilms.

There is limited knowledge of interspecies interactions in biofilm communities. In this study, *Pseudomonas* sp. GJ1, a 2-chloroethanol (2-CE) degrading organism, and *Pseudomonas putida* DMP1, a *p*-cresol degrader, produced distinct biofilms in response to model mixed waste streams comprised of 2-CE and various *p*-cresol concentrations. The two organisms maintained a commensal relationship, with DMP1 mitigating the inhibitory effects of *p*-cresol on GJ1. A triple labeling technique compatible with confocal microscopy was used to investigate the influence of toxicant concentrations on biofilm morphology, species distribution, and exopolysaccharide production. Single species biofilms of GJ1 shifted from loosely associated cell clusters connected by exopolysaccharide to densely-packed structures as *p*-cresol concentrations increased, and biofilm formation was severely inhibited at high *p*-cresol concentrations. In contrast, GJ1 was abundant when associated with DMP1 in a dual species biofilm at all *p*-cresol concentrations, although at high *p*-cresol concentrations it was only present in regions of the biofilm where it was surrounded by DMP1. Evidence in support of a commensal relationship between DMP1 and GJ1 was obtained by comparing GJ1-DMP1 biofilms with dual species biofilms containing GJ1 and *Escherichia coli* 33456, an adhesive strain that does not mineralize *p*-cresol. Additionally, the data indicated that only tower-like cell structures in the GJ1-DMP1 biofilm produced exopolysaccharide, in contrast to the uniform distribution of EPS in the single-species GJ1 biofilm.

Finally, we developed a dual species consortium, the simplest case of a multispecies consortium, for biological detoxification of the insecticide parathion. One member of the consortium was responsible for hydrolyzing the parent compound, yielding two metabolites, para-nitrophenol (PNP) and diethylthiophosphate (DETP). The second member of the consortium was responsible for mineralizing the stable intermediate PNP, a compound classified as a priority pollutant by the United States Environmental Protection Agency. Kinetic parameters required to characterize the biodegradation of parathion were experimentally determined, and were incorporated into a model describing the activity

of the consortium. Additionally, the ability of the consortium to be cultivated as a biofilm was investigated.

CONCLUSIONS: In summary, we have engineered a bacterial strain to degrade organophosphate pesticides and nerve agents. Either used alone or in a consortium with another organism that could degrade an organophosphate hydrolysis product, we were able to demonstrate complete mineralization of the nerve agent.

SIGNIFICANCE: Although this work has focused on the degradation of parathion, we anticipate that the technologies developed here will be applicable to the degradation of other organophosphate contaminants, such as nerve agents, and recalcitrant organic contaminants. The development of rational metabolic engineering technologies for environmental restoration will lead to improved degradation rates, more complete degradation of the contaminant, and bacteria that can compete better in the environment. The application of biodegradation to treat extremely toxic contaminants, such as organophosphate nerve agents, may necessitate such strategies.

PATENT INFORMATION: N/A

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